Saskatchewan
Environment
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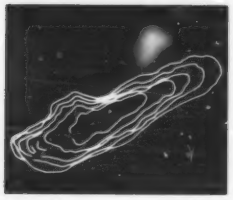
Management

Chemical Marking of Walleye Otoliths

Fish and Wildlife Technical Report 01-1



January 2001

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Methods Required to Chemically Mark, Preserve, and Distinguish Tetracycline Fluorescence on Walleye Otoliths (Sagittae)

Fish and Wildlife Technical Report 01 - 1

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January 2001

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Acknowledgements

The authors would like to thank Curtis Kuntz, Richard Orr, Maynard Chen and the staff of the Fish Culture Station for their assistance with this project.

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Methods Required to Chemically Mark, Preserve, and Distinguish Tetracycline Fluorescence on Walleye Otoliths (Sagittae)

Evaluating the success of fish stocking is an important part of fisheries management. To do this, however, it is necessary to distinguish between stocked and naturally recruited fish. The majority of the larval walleye stocked in Saskatchewan are not readily distinguishable from natural stocks and thus the need for a marking technique. Larval fish are difficult to mark and some form of chemical marking or analysis (e.g. laser ablation spectrophotometry) are the only methods of determining the origin of fish stocked in the larval stage. Agencies in the United States have been using chemical marking to distinguish their stocked fishes for a number of years and it was felt that this could be employed in Saskatchewan. This method is the product of a pilot research study to determine the efficacy and suitability of chemical marking. Initial trials were conducted with oxytetracycline, but it was determined that tetracycline was more appropriate for use in Saskatchewan. This procedure offers a cost effective way to chemically mark larval fish with a minimum of mortality. The employment of this technique will allow fishery managers to determine the overall contribution of stocking to the population of a waterbody and thus the effectiveness of stocking programmes.

Application of Technique

This method is for use with walleye (*Stizostedion vitreum* (Mitchill, 1818)) larvae; however similar techniques may be used to mark the larvae of other teleost fishes (the development of bony / calcareous tissue capable of receiving the mark is the critical factor).

Briefly, larval walleye are immersed in a bath of tetracycline solution. The tetracycline binds with calcium in the animals bony tissue and forms a permanent mark. This mark fluoresces under excitation from ultraviolet light. Any bony tissue from a marked animal should display fluorescence in the layer of bone laid down whilst the animal was treated. This mark theoretically lasts for the lifespan of the animal, thus bony structures may be sampled from any life stage and examined for the presence of a mark. In teleosts (bony fishes), the bony structures most frequently extracted and examined for mark presence are the sagittae (otoliths). This paper describes the techniques for sagittal extraction and examination; although other bony tissue could be processed in a similar fashion.

As with any such procedure, this should only be undertaken by qualified staff (particularly with reference to the handling of larval fish and the operation of ultraviolet illumination equipment).

(A) Marking and Transporting Larval Walleye

Marking is accomplished by immersing three to five day old larvae, for at least 6 hours, in a tetracycline solution of 500 $mg \cdot L^{-1}$. This should not be done in the fall as the annulus (annual growth ring) of an otolith will auto-fluoresce, and could therefore be confused with tetracycline fluorescence. Tetracycline solutions are acidic; therefore some buffering of the bath solution may be required. Buffering agents are not necessary in waters originating throughout southern Saskatchewan. R. Heidinger (pers. comm.) felt that water with a pH near 8 and high alkalinity should have enough innate buffering capacity and experimentation at the Fish Culture Station proved this to be the case. If the application of a buffer becomes necessary, it is important to note that a calcium based buffer cannot be used as the calcium in the buffer will bind to the tetracycline and limit the effectiveness of the marking solution. A sodium phosphate buffer should be used. Since high unionized ammonium will also adversely affect marking, steps should be taken to maximize the quality of holding and transport waters.

(i) Marking Larval Fish

There are two means of marking larval walleye which have been tested in Saskatchewan: mass marking and marking of smaller numbers of animals in plastic transfer containers ('cubes', which larvae are typically transported in). Mass marking is recommended since this allows the fish to be transported in untreated hatchery water that promotes better transport conditions and presumably higher survival. Regardless of the method used; several test containers should be prepared and observed prior to readying the entire group of larvae for marking. As with any procedure involving chemical treatment of animals, adverse physiological or water chemistry reactions are possible. Marking solutions should be monitored for the formation of unusual precipitates or odd water colouration (Note: it may be useful to use a paint chip or colour card of the normal or 'correct' colour as a reference to aid in identifying off-colour solutions). Do not continue with the treatment if this situation persists or undue mortalities are observed.

It is very important that three to five day old larvae are used. Experiments involving the immersion of one to three day post-hatch larvae resulted in poor marking of the otoliths and a lower overall percent of marked larvae. However, four to five day post-hatch larvae were noted to have highly visible marks on 100 percent of their otoliths after examination between three and 50 weeks following immersion.

(ia) Mass Marking (Recommended)

This method involves marking all larvae in a holding tank and, after marking is complete, transferring the animals to transport containers with normal hatchery water. Since the animals will be held for six hours, the marking tank should be equipped with some means of temperature control/regulation and a circulation system. The circulation can not be vigorous or involve direct aeration; violent mixing and aeration tend to promote the formation of harmful precipitates in the tetracycline solution. Select a holding tank suitable for the number of larvae to be marked, generally in the 1,000 L capacity range.

Once the tank mechanics are readied, prepare the solution and mark the animals by:

1) adding the holding water and determining the volume as precisely as possible (within 10 percent error, thus the minimal tetracycline concentration would be 450 mg·L $^{-1}$);
2) dissolving an appropriate amount of tetracycline powder to bring the solution to 500 mg·L $^{-1}$. The solution should take on an amber, not bright yellow, colouration and be free of flock or precipitate;
3) adding buffer, if necessary (as noted previously this step is normally not necessary at the hatchery, or throughout southern Saskatchewan, due to the high pH of the hatchery water). A pH meter is used to determine the necessity to buffer and the amount required. Marking solution should be buffered back to the pH of the animal's rearing water or neutral pH (7), whichever is lower (phosphate buffers are typically weak and are unlikely to raise the pH above neutral; addition of buffer beyond the point where the solution has been neutralized may create a toxic solution);
4) adding three to five day post-hatch larvae, and
5) holding the animals in the marking solution for six hours before transferring the larvae to transport containers.

Transfer the larvae to transport 'cubes' containing normal hatchery/rearing water as per routine hatchery practices.

(ib) Cube Marking

The following is a plastic water container ('cube') immersion technique based on the procedures of Brooks, Heidinger and Kohler (1994).

Each plastic stocking bag should be readied by:

1) adding 5.5 grams of tetracycline powder;
2) adding 11 litres of water. The solution should take on an amber, not bright yellow, colouration and be free of flock or precipitate;
3) adding buffer, if necessary (as noted previously this step is normally not necessary at the hatchery, or throughout southern Saskatchewan, due to the high pH of the hatchery water). A pH meter is used to determine the necessity to buffer and the amount required. Transport/marking solution should be buffered back to the pH of the animal's rearing water or neutral pH (7), whichever is lower (phosphate buffers are typically weak and are unlikely to raise the pH above neutral; addition of buffer beyond the point where the solution has been neutralized may create a toxic solution);
4) adding 50,000 three to five day post-hatch larvae, and
5) filling the head space with gaseous oxygen (do not aerate the water directly, violent mixing may cause the formation of harmful precipitates).

Note: prior mixing of the overall amount of transport water, tetracycline and any other components in a large tank may be advantageous. The pre-mixed solution can then be drawn off and added to the transport containers as required.

(ii) Transporting Marked Larvae

All cubes should be loaded and transported to the stocking location following standard protocol. Ensure that a stocking report is properly filed and indicates that marking procedures were carried out. At the water body, bags are floated to allow the larvae to acclimatise to ambient temperature. If cube marking is being performed, ensure the larvae have been immersed in tetracycline solution for a minimum of six hours before they are released. Limiting larval mortality is crucial, animals should never be kept in transport containers longer than necessary (whether in marking solution or not).

(iii) Releasing Larvae

Record the following information at time of larval release:

- time of day;
- water temperature of lake;
- location of release (location from shore/depth of water),
- type of habitat at the release site. This should include the type of lake bottom and type of aquatic vegetation that may be present, and;
- the amount of larval mortality, to be expressed as a percent.

Also conduct:

- a vertical plankton haul (if depth > 2 metres), use haul rate of one metre per five seconds, note depth of haul, wash sample from net into a jar with alcohol if identification is to be delayed for any time;
  or
- a horizontal plankton tow (if depth < 2 metres), maintain constant haul rate, record rate and distance, wash sample from net into a jar with alcohol if identification is to be delayed for any time.

Plankton counts are used to establish forage availability for the stocked larvae and contribute to survival estimates for the marked fish.

(B) Handling and Storage of Recaptured Specimens

Generally, juvenile fish should be collected upon reaching at least 7.5 cm in length in order to provide reasonably sized otoliths. Smaller fish, including larvae, can be assessed for marks; this simply requires greater care and patience. Immediately upon collection (by electrofishing, seining, minnow traps, drift nets, etc.) fish should be frozen whole until time of analysis. While in the field, fish may be kept on ice until they can be frozen. Fish samples should not be thawed until the otoliths are to be removed for study.

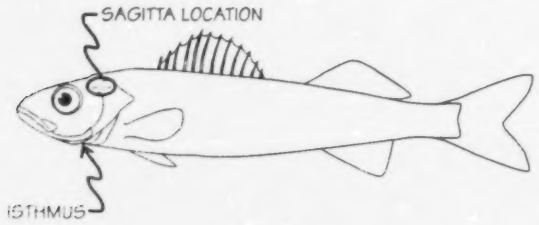
(C) Preparation of Otoliths and Detection of Tetracycline Fluorescence

Otoliths must be examined within 30 days of extraction from the fish. After this period tetracycline marks will have degraded to the point where they are no longer detectable or distinguishable from autofluorescence. R. Brooks (pers. comm.) habitually prepares and examines otoliths for marks within 6 hours of extraction.

Otoliths should be extracted from small fish with the aid of a dissecting microscope. Small otolith extraction techniques are described by Secor et al., 1991. The procedure for removing otoliths from juvenile walleye is as follows (refer to figures):

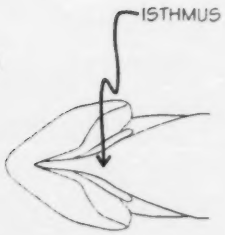
1) Place the fish on its dorsal surface and grasp the isthmus whilst spreading the gill covers apart (Figure A).
2) Firmly grasp the isthmus (Figure B) with a forceps (Figure C) and remove the isthmus and gill tissue. The otoliths (sagittae) should now be readily apparent as indicated in Figure D.
3) Remove the otoliths with the forceps tip (Figure E).
4) Wipe otoliths clean with a Chemwipe or cloth to remove residual fleshy tissue (tissues other than bone can also cause confounding auto-fluorescence).

Extraction of sagittae (otoliths) from juvenile walleye.



A

B



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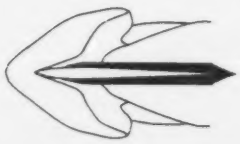
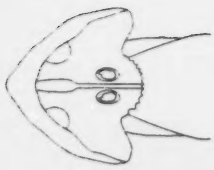


D



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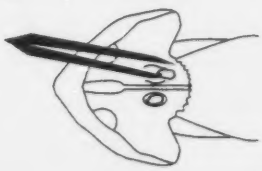
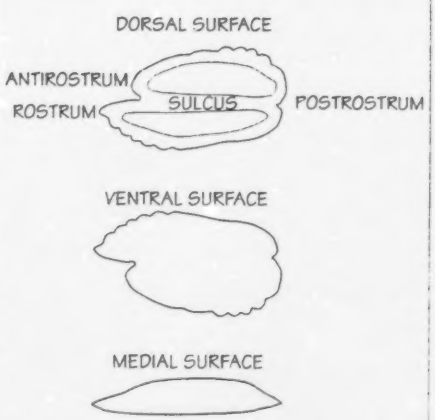


Appearance and features of juvenile walleye sagittae (otoliths).



(i) Mounting of Otoliths

Otoliths from fish collected more than 45 days after immersion likely require grinding down and therefore mounting. Use a cyanoacrylate 'super glue' adhesive (Zap-A-Gap Ca $^{+}$™, KrazyGlue™, etc.) for mounting. Use of thermoplastic cement (epoxy etc.) is not recommended; the heat generated by this type of glue degrades fluorescent marks. Otoliths should be mounted 'cup up' on the ventral side with the sulcus facing upwards. Place a small amount of glue on a regular glass microscopy slide and then gently fix the otolith in place. No more than two otoliths should be fixed to a slide and these should be at either end to facilitate grinding.

Otoliths from fish collected less than 45 days after immersion require no grinding preparation as fluorescence is easily noted from the periphery. These otoliths need not be mounted or fixed to the viewing slide.

(ii) Grinding Down of Otoliths

Grinding down is required for otoliths from fish collected more than 45 days after immersion in order to expose the marked nucleus.

After glue mounting, an otolith should be wet-ground down to the nucleus using 600-grit sandpaper. The paper should be wetted slightly with water (saliva also works well) and set on a flat, solid surface. The slide, with otolith affixed, is moved in a circular motion with light pressure directly on the otolith as it is ground (this helps prevent the bone from fracturing apart). The amount of grinding required depends on the age of the fish. R. Heidinger (pers. comm.) has examined three year old walleye otoliths and found fluorescence after grinding. Grinding should be done slowly, examining the otolith periodically using a compound microscope on low power and under transmitted white (normal spectrum) light. Fluorescent marks will only be visible under incident ultraviolet light when the otolith's entire nucleus becomes visible under transmitted white light. With limited practice, one should be able to quickly grind otoliths down to the nucleus.

(iii) Fluorescence Microscopy

The best system to detect tetracycline fluorescence in otoliths (Baumgaurdner 1991) is a compound microscope with 100 - 400 X magnification, equipped with a 100-W ultraviolet light source, a 450 - 490 nm excitation filter, a 515 nm barrier filter, and a 510 nm dichroic mirror. Fish and Wildlife Branch has the correct filter package and mercury vapour lamp (burner) available for use with a Karl Zeiss compound transmitted light microscope. The scope can be set up for incident light fluorescence microscopy relatively quickly. Contact Kevin Murphy regarding setup, adjustment, maintenance and use of this assembly. *Do not use this assembly without prior instruction.* The UV light is very hazardous to unprotected vision.

Examine specimens with transmitted white light first, adjusting power as necessary, then switch to incident UV light. Fluorescence microscopy must be performed in a dark room free of other UV light sources (sun, fluorescent lights, etc.) which can induce spurious fluorescence in specimens. If very high power (oil immersion lens) is desired, type-b immersion oil must be used as it does not block ultraviolet light and does reduce autofluorescence.

(iv) Appearance of Fluorescent Marks on Otoliths

Tetracycline fluoresces a yellow-gold (amber) colour. The location of tetracycline fluorescence is associated with the daily ring at age of immersion. In a few cases the mark may extend over more than one daily ring. Autofluorescence is typically of a more green or blue colouration (the otoliths examined to date have all been of a greenish hue). R. Heidinger (pers. comm.) indicated that problems arise if the observer is colour blind. This creates difficulty for the observer in distinguishing autofluorescence from tetracycline fluorescence. Those looking for marks for the first time should work with someone with previous experience in order to safeguard against possible difficulties in perceiving fluorescence and/or differentiating auto versus mark fluorescence.

(D) Acquiring and Using Tetracycline

Tetracycline is a drug, and as such falls under the jurisdiction of Health and Welfare Canada. When administered to animals which may be consumed by humans, drugs are approved by the Health Protection Branch, Bureau of Veterinary Drugs, Human Safety Division. Tetracycline is one of the few dugs approved for use with food fish (pers. comm. Prof. H. Ferguson, D.V.M., 16/04/97). As such it may be purchased, for the purposes of marking, from any veterinarian to be administered under their supervision. A written statement explaining the use to which the drug will be put, along with proper identification should accompany any request for purchase. The Fish Culture Station retains the services of a veterinarian and this individual is the logical contact for tetracycline supplies. Tetracycline should be purchased as Tetracycline 1000, a soluble powder composed of tetracycline hydrochloride (HCl) at 1000 $mg \cdot g^{-1}$ concentration (pure).

(E) Contacts

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Calgary
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Appendix 1: Experimental Trials

Various experimental trials were conducted at the Fish Culture Station and the Edenwold Rearing Ponds to test the suitability and efficacy of chemical marking. This is a list of the trials, by year, with some brief explanatory notes regarding each.

1996

Marking experiments were conducted with oxytetracycline, rather than tetracycline. Unlike tetracycline, oxytetracycline was readily available from veterinary suppliers at the time (the opposite of the current situation). Permission was sought for experimental drug use through Agriculture Canada, which was later determined to be unnecessary (see explanation in methods).

Marked walleye were stocked in Dore Lake and the Edenwold Rearing Ponds. Mortalities were reported among those animals transported to Dore Lake (numbers recorded in the stocking reports). There were no mortalities among larvae stocked to the Edenwold Ponds; but subsequently there was exceptionally high mortality among juveniles (thermal stress is suspected). No animals were recovered from the ponds. To date, no otoliths from Dore Lake animals have been checked for the presence of marks.

No buffer was used in these marking trials and aeration was applied to the holding/transport water. Some formation of flock/precipitate was observed and this may have contributed to the mortality of the larvae.

1997

Tetracycline was adopted in 1997. Buffering of the marking solution was tested and shown to be ineffective for the high pH hatchery waters. Innate buffering allowed 15 L of marking solution to reach pH 7.3 (from 8.1) after addition of 7.5 g tetracycline. Addition of sodium phosphate buffer did not appreciably increase the pH of the marking solution, even after adding 1.5 L (which in itself would be detrimental to the larvae). Buffering was abandoned and not used during the marking of fish stocked to the rearing ponds.

Marked fish were stocked in the Edenwold Rearing Ponds. Two ponds, 4 and 5, were stocked with marked walleye larvae. Mortality (estimated at 27%) was reported among these larvae, which was attributed to a 14 degree difference in transport and receiving waters (no mortality was observed up to the point of acclimation in the receiving waters). Juvenile fish were recovered from the ponds and 100 (50 marked and 50 unmarked) were frozen for later examination. Sadly, the frozen samples disappeared from storage; thus the efficacy of tetracycline marking could not be determined in 1997.

1998

The marking and stocking of larvae was conducted as for 1997. No mortalities among the larvae were reported. Juvenile fish were recovered and samples mercifully remained in the researcher's custody long enough to be examined. Otoliths extracted from marked animals all displayed marks. Ten samples were sent to Prof. Brooks who confirmed the presence of marks.

1999

The marking of larvae was conducted as for 1997 and 1998. Five ponds at Edenwold were stocked with marked walleye larvae. No mortalities among the larvae were reported. Juvenile fish were recovered from the ponds and samples examined. Otoliths extracted from marked juveniles all displayed marks. Condie Reservoir received 6,800 marked juveniles. We intend to sample this population over the next decade to establish the permanency or lifespan of the mark in treated animals.

2000

Initial trials were conducted by hatchery staff to determine feasibility of mass marking. A 1,000 L solution was prepared and marked larvae were then transferred to the Edenwold Rearing Ponds. Samples were taken periodically from the ponds to aid in establishing a cut off point for mark visibility in unprepared otoliths. The marks fade around 48 days, with otoliths from more mature animals requiring grinding to make the mark visible. All of the otoliths displayed marks, confirming the suitability of the hatchery trials for mass marking.